

**The IKK $\alpha$ -dependent signaling pathway is required  
in stromal cells and B lymphocytes for p52:RelB activation, FDC maturation  
and germinal center formation**

Giuseppina Bonizzi<sup>1</sup>, Magali Bebien<sup>1\*</sup>, Kirsten Elizabeth Johnson<sup>2\*</sup>, Dennis C.  
Otero<sup>2</sup>, Yixue Cao<sup>1</sup>, Michael Karin<sup>1,4</sup> and Robert C. Rickert<sup>2</sup>

Running title: IKK $\alpha$  is required in stromal cells and B lymphocytes

<sup>1</sup>Laboratory of Gene Regulation and Signal Transduction  
Department of Pharmacology, School of Medicine,  
<sup>2</sup>Division of Biological Sciences and UCSD Cancer Center  
University of California, San Diego  
9500 Gilman Drive, La Jolla, CA 92093-0636

NAGRO

C. J. DelNagro

<sup>4</sup>To whom correspondence should be addressed:

Phone: 858-534-1361

Fax: 858-534-8158

e-mail: karinoffice@ucsd.edu

## Abstract

I $\kappa$ B Kinase (IKK) $\alpha$  is required for activation of an alternative NF- $\kappa$ B signaling pathway based on processing of the NF- $\kappa$ B2/p100 precursor protein, which associates with RelB in the cytoplasm. This pathway, which activates RelB:p52 dimers, is required for induction of several chemokine genes needed for organization of secondary lymphoid organs. We investigated the basis for the IKK $\alpha$ -dependence of the induction of these genes in response to engagement of the lymphotoxin  $\beta$  receptor (LT $\beta$ R). Using chromatin immunoprecipitation we found that the promoters of organogenic chemokine genes are recognized by RelB:p52 dimers and not RelA:p50 dimers, the ubiquitous target for the classical NF- $\kappa$ B signaling pathway. Furthermore, we identified in these promoters a novel type of NF- $\kappa$ B binding site that is preferentially recognized by RelB:p52 dimers. This site, which is also present in the 5' regulatory region of other IKK $\alpha$ -dependent genes, links induction of organogenic chemokines and other important molecules to activation of the alternative pathway.

## Introduction

The canonical NF- $\kappa$ B signaling pathway, which is activated by proinflammatory cytokines and pathogen associated molecular patterns (PAMPs), depends on inducible degradation of specific inhibitors, I $\kappa$ Bs, which retain different NF- $\kappa$ B dimers in the cytoplasm (Ghosh and Karin 2002). This pathway is largely dependent on IKK $\beta$ , a component of a complex that also contains the IKK $\alpha$  catalytic subunit and the IKK $\gamma$ /NEMO regulatory subunit (Rothwarf and Karin 1999). In this pathway, IKK $\beta$  phosphorylates I $\kappa$ Bs at N-terminal sites to trigger their ubiquitin-dependent degradation and induce nuclear entry of NF- $\kappa$ B dimers (Karin and Ben-Neriah 2000). Recently, a second NF- $\kappa$ B activation pathway based on regulated processing of the NF- $\kappa$ B2/p100 precursor protein was identified (Senftleben et al. 2001; Xiao et al. 2001). NF- $\kappa$ B2/p100 consists of an N-terminal Rel homology domain (RHD), common to all NF- $\kappa$ B proteins, and an inhibitory I $\kappa$ B-like C-terminal domain (Ghosh et al. 1998). The presence of the latter prevents nuclear translocation of p100 and its partners.

IKK $\alpha$  and IKK $\beta$ , activate at least a dozen NF- $\kappa$ B dimers, composed of five subunits (Ghosh and Karin 2002). While the mechanisms of NF- $\kappa$ B activation are well understood (Ghosh and Karin 2002), the generation of biological specificity by this complex system is more enigmatic (Pomerantz and Baltimore 2002). Mouse mutagenesis experiments indicate that IKK $\beta$  activates the classical NF- $\kappa$ B pathway, represented by RelA:p50 dimers, in response to stimuli such as tumor necrosis factor (TNF) $\alpha$  (Li et al. 1999; Chen et al. 2003). The mechanisms by which IKK $\alpha$  regulates cytokine-induced gene expression are more obscure and controversial (Israel 2003). *In vivo* analysis revealed that IKK $\alpha$  activates an alternative NF- $\kappa$ B pathway based on processing of NF- $\kappa$ B2/p100 and release of RelB:p52 dimers in response to

LT $\alpha$ / $\beta$  trimers (Dejardin et al. 2002) and other TNF family members (Claudio et al. 2002; Kayagaki et al. 2002). This pathway is required for secondary lymphoid organogenesis and induction of genes involved in this process, but has no apparent role in TNF $\alpha$ -induced functions (Senftleben et al. 2001; Dejardin et al. 2002). We have used mice in which IKK $\alpha$  was rendered inactivateable (Cao et al. 2001) to study the mechanism responsible for selective gene induction by the alternative NF- $\kappa$ B signaling pathway. Using primary cultures of splenic stromal cells and bone marrow-derived myeloid dendritic cells (BMDCs), we found that generation of gene induction specificity by IKK $\alpha$  depends on selective activation of RelB:p52 dimers, which recognize a unique type of NF- $\kappa$ B binding site. This novel cis element is responsible for rendering the induction of organogenic chemokines IKK $\alpha$ -dependent. We also found similar sites in several other genes that are IKK $\alpha$ -dependent.

## Results and Discussion

### FDC maturation and altered B/T-cell segregation is IKK $\alpha$ -dependent.

Lethally irradiated mice reconstituted with *Ikk $\alpha$ <sup>-/-</sup>* hematopoietic progenitors revealed a role for IKK $\alpha$  in late B-cell maturation, splenic organization and germinal center (GC) formation (Kaisho et al. 2001; Senftleben et al. 2001). However, embryonic lethality precludes the use of *Ikk $\alpha$ <sup>-/-</sup>* mice to identify functions for IKK $\alpha$  in other cell types. Homozygous knock-in mice expressing an IKK $\alpha$  variant that cannot be activated (*Ikk $\alpha$ <sup>AA/AA</sup>* mice) are viable, yet show defective lymphoid organogenesis and GC formation (Senftleben et al. 2001). To identify the cells in which IKK $\alpha$  acts to control secondary lymphoid organogenesis, reciprocal bone marrow chimeras were generated between *Ikk $\alpha$ <sup>AA/AA</sup>* and WT mice. The chimeric mice were challenged

with a T-cell dependent antigen, sheep red blood cells (SRBC), and sacrificed 7 days later. Using an antibody against CD35, we examined formation of mature follicular dendritic cells (FDC), a cell type derived from mesenchymal stromal cells that is important for GC formation (Fu and Chaplin 1999). FDC maturation was impaired in *Ikkα<sup>AA/AA</sup>* recipients reconstituted with WT bone marrow, whereas a mature FDC network formed in WT recipients reconstituted with *Ikkα<sup>AA/AA</sup>* bone marrow (Fig 1A). These results suggest that IKKα acts in stromal cells of the spleen to induce their maturation into FDCs.

Another aspect of proper splenic development is segregation of B and T-lymphocytes to the follicles and the peri-arterial lymphatic sheath (PALS), respectively. WT chimeras reconstituted with *Ikkα<sup>AA/AA</sup>* bone marrow, but not *Ikkα<sup>AA/AA</sup>* mice reconstituted with WT bone marrow, exhibited normal B- and T- cell segregation (Fig 1B). These results also point to a critical action of IKKα in stromal cells, which control splenic microarchitecture through production of organogenic chemokines that dictate cell migration and positioning (Ansel and Cyster 2001), other than the hematopoietic compartment as previously assumed (Kaisho et al. 2001; Senftleben et al. 2001). Critical organogenic chemokines for spleen development include ELC and SLC, ligands for the chemokine receptor CCR7, BLC, which binds CXCR5 (Forster et al. 1999; Ansel et al. 2000) and SDF-1, which promotes trafficking of both immature and naïve lymphocytes to lymphoid tissues (Kim and Broxmeyer 1999). Previous work revealed that induction of these chemokines in response to engagement of LTβR is defective in *Ikkα<sup>AA/AA</sup>* mice (Dejardin et al. 2002). We extended these observations to SRBC immunized mice (Fig 1C). Based on previous experiments, we examined the expression of the different genes at 48 hrs post-immunization. While induction of the mRNAs for BLC, ELC, SLC and SDF-1 was readily detected in WT spleens, these genes were barely induced in the mutant.

The defects shown above are very similar to those exhibited by mice lacking LT $\beta$ R (Fu and Chaplin 1999). The major cell type expressing LT $\beta$ R in the spleen is the stromal cell. To examine the role of IKK $\alpha$  in LT $\beta$ R signaling in splenic stromal cells, as well as in BMDC, which also express LT $\beta$ R (Browning and French 2002), we isolated and cultured these cells from WT and *Ikk $\alpha$ <sup>AA/AA</sup>* mice. Stimulation of WT stromal cells with agonistic anti-LT $\beta$ R antibody (Dejardin et al. 2002) resulted in 4-6-fold induction of BLC, SDF-1, TNF $\alpha$ , VCAM-1 and I $\kappa$ B $\alpha$  mRNAs (Fig 2A). Modest induction of ELC and SLC mRNAs was also observed. Both basal expression and induction of BLC, SDF-1, ELC and SLC mRNAs were defective in *Ikk $\alpha$ <sup>AA/AA</sup>* stromal cells, but induction of TNF $\alpha$ , I $\kappa$ B $\alpha$  and VCAM-1 remained intact or became more efficient. The increased expression of VCAM-1 could be related to the defective nuclear entry of RelB in *Ikk $\alpha$ <sup>AA/AA</sup>* cells (see below), as RelB-deficiency was found to increase the expression of certain inflammatory genes (Xia et al. 1999). By contrast, very little differences in expression of TNF $\alpha$ -inducible genes were found between WT and *Ikk $\alpha$ <sup>AA/AA</sup>* stromal cells (Fig 2A). Unlike anti-LT $\beta$ R, TNF $\alpha$  was a poor inducer of the organogenic chemokines, but was a potent inducer of TNF $\alpha$ , I $\kappa$ B $\alpha$  and VCAM-1.

TNF $\alpha$  induced both rapid and delayed nuclear translocation of RelA in WT and *Ikk $\alpha$ <sup>AA/AA</sup>* stromal cells (Fig 2B). Similar results were obtained using mouse embryo fibroblasts (MEFs), which are related to stromal cells in their mesenchymal origin (see Supplementary Figure). We also extended the analysis to BMDCs, which are of myeloid origin. Induction of RelA nuclear translocation by anti-LT $\beta$ R was also not affected by the *Ikk $\alpha$ <sup>AA</sup>* mutation (Fig 2D). Neither TNF $\alpha$  nor anti-LT $\beta$ R had a significant effect on the subcellular distribution of p50, as this NF- $\kappa$ B subunit was constitutively nuclear (Fig 2D). Both TNF $\alpha$  and anti-LT $\beta$ R induced nuclear

translocation of RelB in WT cells, but only TNF $\alpha$  was capable of sending RelB to the nucleus of *Ikk $\alpha$ <sup>AA/AA</sup>* cells (Fig 2B, D). As expected, only anti-LT $\beta$ R, but not TNF $\alpha$ , stimulated nuclear entry of p52 and this effect was seen only in WT cells (Fig 2B, D). In WT BMDCs, LT $\beta$ R engagement led to induction of SLC, ELC and I $\kappa$ B $\alpha$  mRNAs (Fig 2C). SLC and ELC, however, were not induced in BMDC from *Ikk $\alpha$ <sup>AA/AA</sup>* mice. Again, we found that at least one gene, this time CXCR5, was hyperinducible in mutant cells. These results and the previous genetic analysis of NF- $\kappa$ B2- (Poljak et al. 1999) and RelB- (Weih et al. 2001) deficient mice strongly suggest that *Blc*, *Sdf-1*, *Elc* and *Slc* gene induction requires RelB:p52 nuclear translocation.

To address whether these genes are in fact direct targets for RelB-containing dimers and whether they are also recognized by RelA-containing dimers, we performed chromatin immunoprecipitation (ChIP) experiments (Saccani and Natoli 2002). In splenic stromal cells, anti-LT $\beta$ R induced efficient recruitment of RelB, but not RelA, to the *Blc* and *Sdf-1* promoters (Fig 3A). As previously shown, recruitment of NF- $\kappa$ B subunits to promoter DNA may be detected at earlier time points than revealed by immunoblot analysis of nuclear translocation, due to the increased sensitivity of the ChIP assay (Saccani et al. 2001). Anti-LT $\beta$ R induced recruitment of RelB to target gene promoters was abolished in *Ikk $\alpha$ <sup>AA/AA</sup>* cells. However, TNF $\alpha$ -induced RelB promoter recruitment, which was slower and weaker than the response to anti-LT $\beta$ R, was not affected by the *Ikk $\alpha$ <sup>AA</sup>* mutation (Fig 3A). The response to TNF $\alpha$  may depend on formation of RelB:p50 dimers. As a control we analyzed the same immunoprecipitates for presence of the *Tnf $\alpha$*  and *Vcam1* promoter regions. We found efficient precipitation of both promoter fragments by anti-RelA antibodies and weak or no signal with anti-RelB (Fig 3A). Recruitment of either Rel protein to these promoters was not IKK $\alpha$ -dependent. We also examined recruitment of the large subunit of RNA polymerase II (Pol II). Importantly,

recruitment of Pol II to the *Blc* and *Sdf-1* promoters correlated with recruitment of RelB and was seen only in anti-LT $\beta$ R stimulated WT cells, while recruitment of Pol II to the *Vcam1* and *Tnf $\alpha$*  promoters was IKK $\alpha$ -independent (Fig 3A). In BMDC, treatment with anti-LT $\beta$ R induced efficient recruitment of RelB, but not RelA, to the *Elc* and *Slc* promoters (Fig 3B). No recruitment of RelA was observed. By contrast, both RelB and RelA were recruited to the *I $\kappa$ B $\alpha$*  promoter in response to either TNF $\alpha$  or anti-LT $\beta$ R, but neither response was IKK $\alpha$ -dependent (Fig 3B). As observed for RelB, the LT $\beta$ R-induced recruitment of Pol II to the *Slc* and *Elc* promoters was IKK $\alpha$ -dependent (Fig 3B).

Selective recruitment of RelB-containing NF- $\kappa$ B dimers to the *Blc*, *Sdf-1*, *Elc* and *Slc* promoters could reflect, previously unknown, intrinsic differences in sequence selectivity between RelB- and RelA-containing dimers. To examine this possibility, we analyzed binding of NF- $\kappa$ B proteins to the *Blc* and *Elc* promoters. In this experiment we used recombinant NF- $\kappa$ B proteins to generate NF- $\kappa$ B dimers of known composition. Several  $^{32}$ P-labeled probes were derived from the 700 base pair (bp) proximal region (-688 to +12) of the *Blc* promoter, contained within the ChIP primer set (Fig 4A). One of the probes, from -191 to -20, exhibited strong binding to recombinant RelB:p52 and weak binding to RelA:p50 dimers (data not shown). Several other probes (from -770 to -460, -460 to -380 and -380 to -200, as well as -770 to -980) did not bind either dimer (data not shown). To narrow down the sequence responsible for RelB:p52 binding we generated a shorter probe (Probe 1) covering the region from -191 to -64. This probe exhibited very strong binding to recombinant RelB:p52 and only weak binding to RelA:p50 (Fig 4B). On the other hand, the RelA:p50 and RelB:p52 dimers exhibited little differences in their ability to bind a consensus  $\kappa$ B probe, whereas a 200 bp probe (Probe 2) derived from the far 5' upstream region (-1900 to -1700) of the *Blc* gene was preferentially



recognized by RelA:p50 (Fig 4B). Probe 1 (-191 to -64) contains only one potential NF- $\kappa$ B binding site. We synthesized two overlapping smaller probes containing this site (Fig 4C) and used them to examine binding of RelA:p50, RelB:p52, as well as RelB:p50. Both probes, which contained the sequence 5'-GGGAGATTTG-3', were efficiently recognized by RelB:p52 and only weakly by RelA:p50 (Fig 4B and data not shown). Binding of RelB:p50 to these probes was barely detectable. In all cases, the detected protein-DNA complexes were specific as indicated by competition experiments (data not shown).

To identify whether another IKK $\alpha$ -dependent chemokine gene contains a similar sequence, we used the Trafac server (Jegga et al. 2002), which identifies ortholog conserved transcription factor binding sites, to examine the human and rodent *Elc* genes. The putative binding sites were first identified using the MatInspector program (Professional Version 4.3,2000) that utilizes a database of eukaryotic transcription factor binding sites (Jegga et al. 2002). This procedure identified a very similar sequence to the *Blc*- $\kappa$ B site at positions -64 to -50 of the *Elc* genes (Fig 4C). This site, termed the *Elc*- $\kappa$ B site, was also preferentially recognized by RelB:p52 dimers (Fig 4B).

We next used MEFs, which unlike the related stromal cells are amenable to transfection (G.B. and M.B., unpublished results), to examine the function of the RelB:p52 specific sites. Stimulation of WT MEFs with either TNF $\alpha$  or  $\alpha$ -LT $\beta$ R-induced DNA binding activities recognized by the consensus  $\kappa$ B site (Fig 5A). Using the *Blc*- $\kappa$ B and *Elc*- $\kappa$ B sites as probes, we detected induced DNA binding activity only in WT MEFs stimulated with anti-LT $\beta$ R (Fig 5A). This activity was not induced in *Ikk $\alpha$ <sup>-/-</sup>* MEFs. Similar results were obtained in BMDCs analyzed with the *Elc*- $\kappa$ B probe (Fig 5B). Next, we cloned three copies of either the consensus  $\kappa$ B site, the *Blc*- $\kappa$ B site or an inactive version of the latter (*mBlc*- $\kappa$ B) upstream to a minimal

SV40 promoter driving a luciferase reporter and transfected the constructs into WT and *Ikkα*<sup>-/-</sup> MEFs. Whereas the consensus κB site conferred inducibility by either TNFα or anti-LTβR, the *Blc*-κB site conferred an efficient response to anti-LTβR but only a weak response to TNFα (Fig 5C). The mutated *Blc*-κB site was inactive. While the consensus κB site was equally active in WT and *Ikkα*<sup>-/-</sup> MEFs, the *Blc*-κB site no longer conferred anti-LTβR responsiveness in *Ikkα*<sup>-/-</sup> MEFs (Fig 5C). Using the intact *Blc* promoter fused to a luciferase reporter we found efficient induction by anti-LTβR in WT but not in *Ikkα*<sup>-/-</sup> MEFs. This response was dependent on integrity of the *Blc*-κB site and even its conversion to a consensus κB site attenuated the response to anti-LTβR (Fig 5C). The *Elc* promoter also exhibited preferential activation by anti-LTβR that was IKKα-dependent.

To further examine the relevance of the RelB:p52 selective binding site, we conducted a pattern search with two strings, namely AGGAGATTTG (*Elc*-κB) and GGGAGATTTG (*Blc*-κB) using the Trafac server and the BlastZ algorithm (<http://bio.cse.psu.edu>). Closely similar (at least 8/10 identity) sites were detected within 5 kb upstream to the start sites of the *Sdf-1* and *Baff* genes (Fig 5D) whose expression is known to be *Ikkα*-dependent (Dejardin et al. 2002) (Figs 1C, 2A, 5E). We also detected similar and evolutionary conserved sites with the same region of several other genes, whose IKKα-dependence was previously unknown. RT-PCR analysis revealed that two of these genes, *Rxra* and *Irf3*, coding for important transcription factors, were induced in stromal cells in response to anti-LTβR in a manner dependent on IKKα (Fig 5E).

Two distinct pathways leading to selective activation of RelA:p50 and RelB:p52 dimers, dependent on IKKβ or IKKα, respectively, were identified (Ghosh and Karin 2002). Each pathway has distinct biological functions (Li et al. 1999; Senftleben et al. 2001; Chen et al.

2003), that could be mediated in part through selective gene activation (Dejardin et al. 2002). How this occurs was previously unknown. We now show in two different cell types, splenic stromal cells and BMDC, that IKK $\alpha$  is required for induction of four genes encoding chemokines critical for organogenesis of the spleen and maintenance of its microarchitecture because these genes are selectively recognized by RelB-containing dimers, most likely RelB:p52. These genes are preferentially activated by engagement of LT $\beta$ R and are only weakly by TNF $\alpha$ . Whereas the TNF $\alpha$  response is IKK $\alpha$ -independent, the response to LT $\beta$ R engagement is strictly IKK $\alpha$ -dependent, because of two events. First, RelB:p52 dimers have to enter the nucleus, a process dependent on IKK $\alpha$ -mediated p100 processing (Dejardin et al. 2002; Yilmaz et al. 2003). Second, RelB:p52 dimers are selectively recruited to the IKK $\alpha$ -dependent gene promoter. The selective recruitment of RelB to the *Blc* and the *Elc* promoters is likely to depend on a novel  $\kappa$ B site that is preferentially recognized by RelB:p52 dimers. This unique sequence specificity is entirely consistent with sequence differences between the DNA binding loops of RelA and RelB, but was previously unknown (Ghosh et al. 1995). It is certainly possible, however, that additional factors may contribute to selective IKK $\alpha$ -dependent gene activation and that IKK $\alpha$  may also be responsible in certain cell types for activation of the canonical NF- $\kappa$ B pathway (Cao et al. 2001) or for potentiating its ability to activate transcription (Anest et al. 2003; Israel 2003; Yamamoto et al. 2003). Nonetheless, an important mechanism responsible for selective gene activation through the IKK $\alpha$ -dependent alternative NF- $\kappa$ B signaling pathway is based on specific recruitment of RelB:p52 dimers to target gene promoters. Sites similar to the RelB:p52 selective  $\kappa$ B site were detected in the 5' regulatory region of three other genes whose expression was found to be IKK $\alpha$ -dependent.

What is the purpose of the functional separation between the two NF- $\kappa$ B signaling pathways? The IKK $\alpha$ -dependent organogenic chemokines optimize adaptive immunity through proper organization of secondary lymphoid organs. By contrast, IKK $\beta$  is mostly involved in inflammatory and innate immune responses. Thus IKK $\beta$ -mediated NF- $\kappa$ B signaling is responsible for rapid responses to infection and injury, that require recruitment of immune cells out of lymphoid organs to sites of infection. This response depends on pro-inflammatory chemokines, such as MIP-1, MCP-1 and RANTES, which are induced by the canonical NF- $\kappa$ B signaling pathway (Alcamo et al. 2001). The arrival of antigens to secondary lymphoid tissues from distal sites of infection and their processing, presentation and recognition require coordinated activity of DC, macrophages, T cells and B cells, whose recruitment to secondary lymphoid organs depends on IKK $\alpha$ -regulated organogenic chemokines. Premature expression of such chemokines would compromise the immediate anti-microbial response as it may abort the emigration of immune cells to the periphery. It is, therefore, logical that expression of organogenic chemokines would not be induced through the canonical NF- $\kappa$ B signaling pathway. Consistent with its delayed function in adaptive immunity, activation of the alternative NF- $\kappa$ B signaling pathway is slower than the canonical NF- $\kappa$ B signaling pathway and seems to depend on prior activation of the latter (Dejardin et al. 2002). The dependence of the two pathways on distinct but related protein kinases and transcription factors allows for both functional integration and kinetic separation.

## **Materials and Methods**

### **Primary cell cultures**

Stromal cell cultures were established from spleens of WT and *Ikkα<sup>AA/AA</sup>* mice as described (Skibinski et al. 1998). Spleens were gently ground and released cells cultured in DMEM supplemented with heat-inactivated FCS (Invitrogen, Carlsbad, Ca). After one week, non-adherent cells were removed, adherent cells were washed twice with PBS and cultured one more week in DMEM/FCS. Absence of contaminating myeloid and lymphoid cells was verified by flow cytometry (FACSCalibur, Becton Dickinson). Stromal cells are uniformly positive for ICAM-1 (data not shown). BMDCs were cultured as described (Wu and Hwang 2002).

### **Adoptive transfers**

Bone marrow cells ( $3-4 \times 10^6$  cells per mouse) were isolated from femurs of WT or *Ikkα<sup>AA/AA</sup>* mice and injected intravenously into lethally irradiated recipients. Mice were H-2 matched and, in the case of *Ikkα<sup>AA/AA</sup>*, were from the F3-F5 backcross to C57Bl/6. Mice were provided antibiotics in drinking water and sacrificed 6-8 weeks post reconstitution. When indicated, mice were immunized i.p. with SRBC (Colorado Serum Company, Denver, Co) 7 days prior to sacrifice (Poljak et al. 1999).

### **Immunohistochemical analysis**

Cryosections (8 – 10 μM) of spleen were prepared, dried and fixed with acetone before immunohistochemical analysis (Poljak et al. 1999; Weih et al. 2001). Staining reagents were: ER-TR9 (RDI, Flanders NJ), FDC-M2 (ImmunoKontakt, UK), BM-8-bio (RDI), ICAM-1 (Santa-Cruz Biologicals, Ca), MOMA-1 (FITC Calbiochem), MAdCAM (clone MECA-3670), CD11c-bio (clone HL-3), B220, and CD35-bio (clone 8C12) (all from BD Pharmingen). Immunocomplexes were detected using species-specific secondary reagents. Sections were viewed by immunofluorescence microscopy (HM505E Microm Inc, Walldorf, Germany) and

images captured with a digital camera (Nikon E800 Scope with Spot Diagnostics Digital Camera, A.G. Heinze Inc., Lake Forest, Ca).

### **Electrophoretic Mobility Shift Assay and Immunoblots**

Nuclear and cytoplasmic extracts were prepared and analyzed for levels of NF- $\kappa$ B subunits and DNA binding activity (Bonizzi et al. 1999; Senftleben et al. 2001). Recombinant NF- $\kappa$ B subunits (not full length proteins) were produced in *E.coli* and purified as described (Chen et al. 1999). Anti-p52/p100 polyclonal antibody was generously provided by J. Hiscott (McGill University). All other antibodies and immunoblotting procedures were described (Senftleben et al. 2001).

### **Real Time PCR analysis and Chromatin Immunoprecipitation Assay (ChIP)**

RT-PCR was performed using a PE Biosystems 5700 thermocycler following the SyBr Green<sup>TM</sup> protocol. Briefly, 12 ng of total cDNA, 50 nM of each primer and 1x SyBr Green<sup>TM</sup> mix were used in a total volume of 25  $\mu$ l. All values were standardized to that of cyclophilin mRNA. Primer sequences are available upon request. ChIP assays were as described (Saccani and Natoli 2002). Polyclonal antibodies to p65 (C-20), RelB (C-19) and Pol II (N-19) were from Santa Cruz. The sequences of the promoter-specific primers (*Blc* +12 to -688, *Sdf-1* +22 to -678, *Vcam-1* +30 to -640, *I $\kappa$ b $\alpha$*  +20 to -340, *Tnf $\alpha$*  +20 to -545) and a detailed experimental protocol are available upon request.

### **Acknowledgments**

We thank G. Natoli for advice regarding ChIP experiments, M. Delhase, L-C Hsu and A. Hoffmann for discussions, C. Ware and J. Browning for generous gifts of agonistic anti-LT $\beta$ R antibodies and many useful suggestions and discussions, J. Hiscott for the generous gift of anti-p52/p100 antibodies and T. Kato for oligo synthesis. G.B. and Y.C. were supported by postdoctoral fellowships from the Human Frontier Science Project (HFSP) and the State of California Breast Cancer Research Program, respectively. Work was supported by NIH grants to M.K. and to G.G. R.C.R. is an awardee of the Hellman Fellows Program for junior faculty. M.K. is the Frank and Else Schilling American Cancer Society Research Professor.

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## Figure legends

### Figure 1: Stromal cell-derived chemokine production requires IKK $\alpha$ .

(A) Impaired FDC maturation is inherent to the stroma of *Ikk $\alpha$ <sup>AA/AA</sup>* mice. Lethally irradiated WT (n = 6) or *Ikk $\alpha$ <sup>AA/AA</sup>* (n = 6) mice were reconstituted with *Ikk $\alpha$ <sup>AA/AA</sup>* or WT bone marrow, respectively. Spleens were isolated 7 days after immunization with SRBC, cryosectioned and stained with anti-CD35. An FDC network is present in WT mice reconstituted with *Ikk $\alpha$ <sup>AA/AA</sup>* bone marrow, while only peri-follicular rings of CD35<sup>+</sup> immature FDCs are present in *Ikk $\alpha$ <sup>AA/AA</sup>* mice reconstituted with WT bone marrow.

(B) Impaired B/T cell segregation in *Ikk $\alpha$ <sup>AA/AA</sup>* spleens. Lethally irradiated WT (n = 3) or *Ikk $\alpha$ <sup>AA/AA</sup>* (n = 3) mice reconstituted with *Ikk $\alpha$ <sup>AA/AA</sup>* or WT bone marrow cells were immunized and analyzed as above using anti-CD5 (to recognize T cells) and anti-B220 (to recognize B cells). Impaired B/T cell segregation is intrinsic to the *Ikk $\alpha$ <sup>AA/AA</sup>* stroma.

(C) Defective chemokine gene expression in *Ikk $\alpha$ <sup>AA/AA</sup>* spleens. Total splenocytes from naïve and SRBC-immunized (day 2) WT (n = 3) and *Ikk $\alpha$ <sup>AA/AA</sup>* (n = 3) mice were isolated. RNA was extracted and analyzed by RT PCR for expression of mRNAs encoding BLC, SLC, ELC and SDF-1 and two of their receptors (CXCR5, CCR7). The results are averages  $\pm$  SD of three independent experiments normalized to the level of cyclophilin mRNA.

### Figure 2: IKK $\alpha$ is required for LT $\beta$ R-induced RelB:p52 nuclear translocation and chemokine expression in splenic stromal cells and myeloid dendritic cells.

*Ikk $\alpha$ <sup>AA/AA</sup>* stromal cells (A) and BMDC (C) exhibit specific defects in LT $\beta$ R-induced gene expression. Total RNA was extracted from either WT or *Ikk $\alpha$ <sup>AA/AA</sup>* stromal cells or BMDC before and after stimulation with 2  $\mu$ g/ml agonistic anti-LT $\beta$ R antibody or 20 ng/ml TNF $\alpha$ . Gene

expression was analyzed by RT-PCR. Results are averages  $\pm$  SD of three independent experiments normalized to the level of cyclophilin mRNA.

**(B, D)** Nuclear translocation of NF- $\kappa$ B proteins. Stromal cells **(B)** and BMDC **(D)** were stimulated with either anti-LT $\beta$ R antibody or TNF $\alpha$  as indicated. At the indicated time points (hrs), nuclear extracts were prepared and analyzed by immunoblotting for presence of the indicated NF- $\kappa$ B subunits. The levels of histone H2B were examined to control for loading and proper cell fractionation. Contamination with cytoplasmic proteins was monitored by blotting with anti-actin antibody.

**Figure 3: IKK $\alpha$  is required for recruitment of RelB to the *Blc*, *Sdf-1*, *Elc* and *Slc* promoters.**

Primary cultures of stromal cells **(A)** and BMDC **(B)** from WT and *Ikk $\alpha$ <sup>AAA</sup>* mice were left unstimulated or stimulated with TNF $\alpha$  (T) or anti-LT $\beta$ R (L). At the indicated time points (hrs) the cells were collected and recruitment of RelA, RelB and the large subunit of RNA polymerase (Pol II) to the indicated promoter regions was examined by ChIP experiments.

**Figure 4: The *Blc* and *Elc* promoters contain a unique  $\kappa$ B site that is selectively recognized by RelB:p52 dimers.**

**(A)** The sequence of the 700 bp region covering the proximal *Blc* promoter, contained within the ChIP primer set. The RelB-selective  $\kappa$ B site and the TATA box are highlighted. The sequence contained within Probe 1 is indicated by the brackets. **(B)** DNA binding analysis. The different probes were incubated with two different amounts (250 and 500 ng) of the indicated NF- $\kappa$ B dimers and DNA binding was analyzed by EMSA. Note that the NF- $\kappa$ B subunits are not the full

length proteins, thus giving rise to complexes with different electrophoretic mobilities. (C) The sequences of the different  $\kappa$ B sites.

**Figure 5: Selective, IKK $\alpha$ -dependent, activation of the *Blc* and *Elc* promoters by LT $\beta$ R engagement.**

(A-B) Engagement of LT $\beta$ R selectively induces *Blc*- $\kappa$ B and *Elc*- $\kappa$ B binding activities. WT and IKK $\alpha$ -defective MEFs (A) and BMDC (B) were left unstimulated or stimulated with either TNF $\alpha$  or anti-LT $\beta$ R for the indicated times. Nuclear extracts were prepared and incubated with <sup>32</sup>P-labeled probes corresponding to the consensus  $\kappa$ B site (NF- $\kappa$ B) or the *Blc*- $\kappa$ B and *Elc*- $\kappa$ B sites. DNA binding activity was analyzed by EMSA. NF-1 DNA binding activity was measured as an internal control.

(C) Functional analysis of the different  $\kappa$ B sites in the *Blc* and *Elc* promoters. Triple repeats of the consensus  $\kappa$ B (con $\kappa$ B), *Blc*- $\kappa$ B and a mutant *Blc*- $\kappa$ B (m*Blc*- $\kappa$ B) site were cloned upstream to a minimal SV40 promoter (pGL3-Promoter vector, Promega). In addition, the *Blc* (+12 to -688) and *Elc* (+530 to -320) promoter regions were cloned upstream to a luciferase reporter (pGL3-Basic vector, Promega). To determine the importance of the *Blc*- $\kappa$ B site, it was converted by site directed mutagenesis either to an inactive mutant version (m $\kappa$ B) or the consensus  $\kappa$ B (con $\kappa$ B) site. The different plasmids were transfected into WT and *Ikk $\alpha$* <sup>-/-</sup> MEFs. After 6 hrs with TNF $\alpha$  or anti-LT $\beta$ R, luciferase activity was determined. The results are averages  $\pm$  SD of three independent experiments normalized to  $\beta$ -galactosidase activity produced by a cotransfected  $\beta$ -galactosidase expression vector.

(D) Sequences similar to the *Elc*- $\kappa$ B and *Blc*- $\kappa$ B sites are found in the 5' regulatory regions of several other IKK $\alpha$ -dependent genes.

(E) Induction of *Baff*, *Rxra* and *Irf3* is IKK $\alpha$ -dependent. Expression of the indicated RNAs was analyzed by RT-PCR as described above using RNA isolated from non-stimulated and anti-LT $\beta$ R-stimulated stromal cells (*Rxra* and *Irf3*) and BMDCs (*Baff*) of the indicated genotypes.

### **Supplementary data**

**IKK $\alpha$  is required for p100 processing and induction of RelB nuclear entry and DNA binding in response to LT $\beta$ R engagement in fibroblasts.**

(A) IKK $\alpha$ -dependence of p100 processing and nuclear translocation of p52 and RelB. WT and *Ikk $\alpha$ <sup>-/-</sup>* MEFs were stimulated with either 20 ng/ml TNF $\alpha$  or 2  $\mu$ g/ml anti-LT $\beta$ R antibody, as indicated. At the indicated time points (hrs) after agonist addition, cytoplasmic and nuclear extracts were prepared and 10  $\mu$ g of proteins were analyzed for presence of the indicated NF- $\kappa$ B proteins, as well as histone H2B.

(B) IKK $\alpha$ -dependence of RelB DNA binding. WT and IKK $\alpha$  MEFs were left unstimulated or stimulated with either TNF $\alpha$  or anti-LT $\beta$ R for 30 min or 6 hrs as indicated. Extracts were prepared and incubated with a <sup>32</sup>P-labeled  $\kappa$ B probe and DNA binding activity was analyzed by EMSA. To detect the presence of the indicated NF- $\kappa$ B subunits in the DNA bound complexes, supershift analysis was carried out using specific antibodies. The supershifted complexes are indicated by the arrowheads.